JOURNAL OF CLINICAL MICROBIOLOGY, May 1991, p. 962–967
0095-1137/91/050962-06$02.00/0

Vol. 29, No. 5

Comparison of Restriction Enzyme Analysis and Pulsed-Field Gradient Gel Electrophoresis as Typing Systems for *Candida albicans*

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Received 9 July 1990/Accepted 12 February 1991

***Candida* species are an important cause of infection in immunocompromised hosts and the leading cause of nosocomial fungal infections. Study of the epidemiology of *Candida* infection has been difficult because of lack of a reliable typing system. We describe a typing system utilizing contour-clamped homogeneous electric fields (CHEF), which is a modified version of pulsed-field gradient gel electrophoresis, and compared it with restriction enzyme analysis (REA) of genomic DNA. The study was done with 35 *Candida albicans* clinical isolates from separate patients. CHEF and REA were performed on each isolate, and the patterns were compared. The REA procedure revealed 17 strain types while the CHEF procedure was able to distinguish 23 strain types of *C. albicans*. The CHEF technique yields unique patterns of chromosomal bands that can be used to distinguish clinical isolates and demonstrates greater sensitivity than REA.**

The ubiquitous, dimorphic yeast *Candida albicans* is an important cause of vaginitis and nosocomial infections, including life-threatening infections in immunocompromised patients. Advances in chemotherapeutic and transplantation technology and increasing numbers of human immunodeficiency virus-infected patients have resulted in larger numbers of severely immunocompromised hospitalized patients. The incidence of disseminated candidiasis has also increased, as proven by several autopsy series (2, 3, 5, 14, 17, 31). *Candida* species are now the fourth most common organisms isolated in blood cultures. The mortality rate associated with *C. albicans* when cultured from blood is estimated at 70 to 85% (25). For some of the non-*albicans* *Candida* species, fungemia is associated with mortality rates of 90 to 100% (20, 25).

A clear understanding of the epidemiology of *Candida* infection has been difficult because of the lack of a reliable typing system to evaluate strain relatedness. Previous typing systems have relied upon biotyping (28, 48), utilizing various enzyme profiles (7), susceptibility to killer toxins (33), streak morphology (32), resistance patterns and biochemical analysis (28, 45, 46), serological agglutination reactions (45), immunoblotting techniques (21), modifications of older techniques (9), and combinations of several methods. With the advent of molecular genetics, newer typing systems which use comparative analysis of chromosomal DNA (10, 16, 24, 42), ribosomal DNA (11, 22, 35, 36), mitochondrial DNA digested by restriction enzymes (29, 34, 49), or DNA probes (8, 13) have emerged. These techniques have been successfully used to evaluate the strain relatedness of various types of bacteria, viruses, and yeasts (12, 35). Pulsed-field gradient gel electrophoresis (PFGE), is a newer method used in the analysis of very large chromosomal DNA found in yeasts (6, 37). PFGE has already been used in the differentiation of various yeast strains, including *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *C. albicans* (39–41). PFGE has recently been used to analyze strain differentiation in *Pseudomonas aeruginosa* and *Acinetobacter calcoaceticus* infections in urology patients and mechanically ventilated patients (1). This study comparatively evaluated the utility of restriction enzyme analysis (REA) and contour-clamped homogeneous electric field electrophoresis (CHEF), which is a modification of PFGE, as a marker of strain identity for *C. albicans*.

MATERIALS AND METHODS

Yeast strains. Thirty-five clinical isolates from 20 separate patients were evaluated. Seven isolates were from women with recurrent vaginitis, and 28 isolates (11 pharyngeal, 8 perianal, 2 from urine, 1 from sputum, and 6 vaginal) from immunocompromised hospitalized patients were prospectively cultured during a 4-month period in either the bone marrow transplant or medical intensive care unit of a single, large tertiary care hospital. The seven vaginal isolates were from separate women with symptomatic chronic vulvovaginal candidiasis, and the other 28 strains were all colonizing isolates. Nonvaginal strains were isolated from multiple anatomic sites and at separate times from individual patients. All specimens were identified as *C. albicans* by germ tube formation in calf serum and by the Yeast API 20C method (Sherwood Medical, Plainview, N.Y.). Strains were initially isolated on Sabouraud dextrose agar (Difco, Detroit, Mich.), stored in a 1:1 mixture of brain heart infusion broth (Difco) and glycerol (Sigma, St. Louis, Mo.), and frozen at −70°C until analysis.

Genomic DNA preparation. For DNA isolation, yeast cells were grown on Sabouraud agar plates for 24 h at 30°C. Single colonies were then inoculated into 200 ml of YEPD broth (2 g of yeast extract, 2 g of peptone [Difco], 2 g of glucose) and incubated in a water bath at 30°C for 36 h. The method used to prepare the DNA was that described by Petes et al. (30), with the following modifications. The DNA was pelleted at 16,000 × *g* for 10 min, suspended in 5.0 ml of 0.2 M Tris-HCl (Sigma)–5% β-mercaptoethanol (Sigma), and incubated in a water bath at room temperature for 30 min. After incubation, 10 ml of 1 M sorbitol–0.004 M potassium phosphate (Sigma)

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was added to the cell suspension and pelleted at 16,000 × *g* for 10 min. The cells were then suspended in 2.5 ml of sorbitol-potassium phosphate, and Zymolyase 20T (ICN Biomedical, Lisle, Ill.) was added to promote spheroplast formation at a ratio of 80 U/g of cell weight as described by Torres-Bauza and Riggsby (43). Spheroplasts were then lysed by using a solution of 0.2 M sodium chloride–0.1 M EDTA–5% sodium dodecyl sulfate (Sigma)–50 mM Tris (pH 8.5)–20 μg of pronase (Sigma) and incubated at 60°C for 30 min in a water bath. Nucleic acids were extracted by using a saturated solution of 10 mM Tris–1 mM EDTA (pH 8.4)–phenol–chloroform in a ratio of 25:25:24 and then precipitated with 8 ml of cold ethanol. The suspension was then frozen for 1 h at −70°C. The nucleic acids were then centrifuged at 12,000 × *g* for 10 min and dried in a vacuum oven at room temperature for 30 min. The dried DNA was then suspended in 1 ml of TE buffer (1 mM Tris, 1 mM EDTA, pH 8.0) and reprecipitated with 40 μl of 3 M potassium acetate. Two volumes of cold ethanol was added, and then the mixture was frozen either for 1 h at −70°C or overnight at −20°C. The specimens were then centrifuged again at 12,000 × *g* for 10 min and allowed to dry in a vacuum oven for 30 min at room temperature. The DNA was then suspended in 100 μl of TE buffer in microcentrifuge tubes and stored at 4°C until electrophoresis. For electrophoresis of samples, 40 to 50 μg of DNA was digested with 4 μl of restriction endonucleases *Eco*RI and *Msp*I (BRL, Gaithersburg, Md.) as recommended by the manufacturer. The DNA yield from the lysis procedure was 0.8 to 1.0 mg/200 ml of cell culture. Approximately 40 to 50 μg of DNA was used for each REA. This concentration produced the most definitive patterns with the least amount of background haziness. The DNA was run on a 0.7% agarose horizontal gel apparatus at 30 V for 16 h. The gel was then stained in a solution of 0.5 μg of ethidium bromide (Sigma) per ml for 15 min and then destained in distilled water for 60 min before being photographed.

Yeast insert preparation. After isolation on Sabouraud agar, single colonies were incubated in 40 ml of YEPD broth and incubated for 36 h at 30°C in a shaking water bath. The DNA was prepared as described by Schwartz and Cantor (37). The following modifications were made. The cell suspension was pelleted at 3,000 × *g* for 5 min, washed in 10 ml of 1 M sodium chloride–0.5 M EDTA (pH 9.0), and pelleted again at 500 × *g* for 10 min. The cells were then suspended in 2.0 ml of NaCl-EDTA solution and placed in a 37°C water bath. Low-melting-temperature 1% agarose (Sigma) was prepared with 2 ml of 125 mM EDTA (pH 7.5) and placed into the cell suspension at 37°C. Zymolyase 20T was then added to the suspension at a concentration of 80 U/g of cells. The cell-agarose-Zymolyase suspension was then distributed by Pasteur-pipette into a premade mold (2 by 1 cm by 1.5 mm; Bio-Rad, Richmond, Calif.) and refrigerated for 60 min at 4°C. The inserts were then placed into equal volumes of 0.5 M EDTA–7.5% β-mercaptoethanol and incubated overnight in a 37°C water bath with gentle shaking. After 12 h (overnight), inserts were removed from the solution and washed once with 5 ml of 50 mM EDTA (pH 7.5). The inserts were then transferred to 10-ml tubes, and 3.3 ml of ESP solution (25 ml of 0.5 M EDTA [pH 9.0], 2.8 ml of 10% sarcosyl [Sigma], 0.5 mg of pronase per 28 ml) was added. The inserts were then incubated overnight at 50°C in a water bath. After 12 h (overnight), the inserts were refrigerated at 4°C for 1 h and then transferred to microcentrifuge tubes, to which 1 ml of 0.5 M EDTA (pH 9.0) was added. The DNA inserts were then stored at 4°C until electrophoresis. The yeast inserts were electrophoresed on a CHEF DR II (Bio-Rad) apparatus in a 0.8% agarose gel with a premade buffer of 0.089 M Tris–0.089 M borate–0.0025 M EDTA (Schwartz-Mann, Cleveland, Ohio) diluted in distilled H_2O to a 0.5 M concentration. The parameters used for PFGE were (i) a 120-s pulse for 24 h at 150 V, (ii) a 300-s pulse for 6 h at 150 V, and (iii) a 300-s pulse for 24 h at 113 V. A constant temperature (12 to 14°C) was ensured by using a chiller water bath and a pump. The gel was subsequently stained in ethidium bromide solution at 0.5 μg/ml for 15 min and then destained in distilled water for 60 min before being photographed. Differences between isolates (strain types) was determined by visual comparison of the DNA patterns. The variations in these patterns formed the basis of our groups. Strain differentiation by CHEF was achieved by visual comparison of the more variable chromosomal bands.

TABLE 1. Comparative analysis of *C. albicans* strain types based on restriction enzyme patterns

*Eco*RI group	No. of isolates	No. in the following *Msp*I group:					
		a	b	c	d	e	f
A	12	4	1	2	3	1	1
B	11	6	1	2	2		
C	2	2					
D	3	2	1				
E	4	2	2				
F	3	2	1				

RESULTS

Restriction enzyme patterns of *C. albicans* DNAs. Thirty-five strains were evaluated by REA. Distinguishable restriction enzyme patterns of chromosomal DNAs were produced by using endonucleases *Msp*I and *Eco*RI. Agarose gel electrophoresis produced over 100 fragments, with fragment sizes varying from 2 to 20 kb. The REA results are shown in Table 1. Digestion of chromosomal DNA with *Eco*RI produced six strain types. Group A consisted of 12 isolates, group B had 11 isolates, group C consisted of 2 isolates, group D had 3 isolates, group E had 4 isolates, and group F had 3 isolates. The restriction enzyme patterns obtained with *Msp*I were more definitive. With *Msp*I, further differentiation of the six strain types produced by *Eco*RI digestion was obtained and 17 strain types were observed. Group A was further divided into six subgroups, group B was divided into four subgroups, group C had two subgroups, and groups D, E, and F were divided into two subgroups each when their isolates were subjected to *Msp*I digestion. Figure 1 shows purified chromosomal DNA isolated from *C. albicans*. Lanes B to E contained isolates digested with *Eco*RI and have identical patterns; all of the isolates were from separate patients. Lanes F to I contained the same isolates digested with *Msp*I, showing different patterns, indicating that they are all different strain types. The restriction enzyme patterns were distinguished by using primarily the repeated sequences or the high-molecular-weight fragments, which were the most variable in our isolates and were the more intensely staining bands. Reproducibility was found with repeated testing of triplicate replicates. All isolates retained the same restriction patterns with repeated testing. Stability of the restriction patterns was determined by repeated evaluation of the isolates after passage through multiple generations.

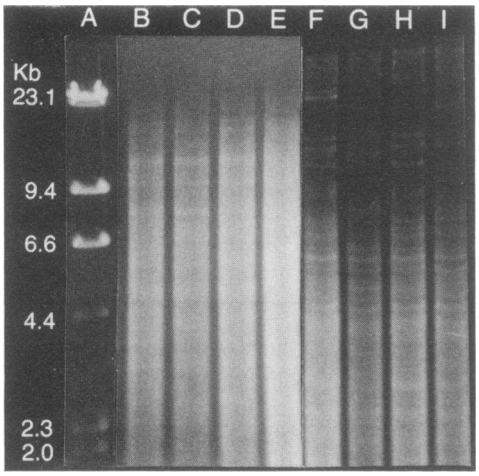

FIG. 1. Chromosomal DNAs from separate clinical *C. albicans* isolates. Lane A contained bacteriophage lambda digested with *Eco*RI. Lanes B to E contained DNAs digested with *Eco*RI. Lanes F to I contained the same DNAs digested with *Msp*I.

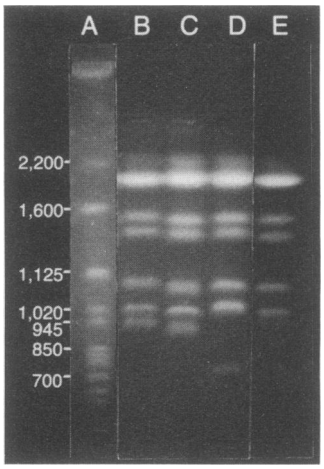

FIG. 2. CHEF electrophoresis of genomic DNAs from separate *C. albicans* clinical isolates. Lane A contained *S. cerevisiae* DNA, which was used as a control.

CHEF patterns of *C. albicans* DNAs. All 35 clinical isolates were also subjected to PFGE by the CHEF method. CHEF yielded definitive patterns with 6 to 8 bands, ranging in size from 460 to 2,000 kb, corresponding to the electrophoretic karyotype of *C. albicans*. With the CHEF method, there were 23 separate strain types of *C. albicans* (Table 2). Ten groups had more than one isolate (group B had five, and groups A, F, K, N, O, P, S, T, and U each had two isolates). Groups with more than one isolate represent specimens from the same patients but different anatomical locations. Similarities among all isolates were noted in bands 1, 2, 3, 5, and 6. Differences between isolates were found in bands 4 and 7, with some *Candida* species having an additional band at a lower molecular weight. Figure 2 shows the CHEF patterns of four separate clinical isolates. All of the isolates shown in Fig. 2 were the same by REA using *Msp*I and *Eco*RI but were different by the CHEF method. Reproducibility of CHEF was established by repeated testing, which showed identical patterns. Stability was proven by the identical patterns shown when the isolates were passed through multiple generations. All of the repeated CHEF patterns were unchanged for each of the isolates. To test for the longevity of the prepared yeast inserts, we repeated electrophoresis of the same strains at 2-, 3-, and 4-month intervals. There were no differences between newer yeast inserts and similar older isolates that had been previously prepared. Comparison of 21 specimens isolated from multiple sites from eight individual patients revealed that they were the same strain type by REA and CHEF. Four patients had two different strain types at the various anatomical sites from which isolates were cultured. Separate patients that were epidemiologically unrelated had different strain types.

DISCUSSION

Advances in medical therapy of neoplastic diseases, together with new invasive surgical procedures and burn therapy, have not only increased the survival of patients but have, at the same time, contributed to an increasing rate of

TABLE 2. Comparative analysis REA and CHEF for typing of *C. albicans*

REA subtype	No. of isolates	No. with the following CHEF subtype:																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Aa	4	1		1		1	1																	
Ab	1								1															
Ac	2		1		1																			
Ad	3									1									1	1				
Ae	1													1										
Af	1							1																
Ba	6		3								1											1		1
Bb	1																				1			
Bc	2											1											1	
Bd	2		1									1												
Ca	2	1											1											
Da	2														2									
Db	1										1													
Ea	2															2								
Eb	2																2							
Fa	2																	2						
Fb	1																	1						

nosocomial infections (4, 38). Among nosocomial infections, fungal infections, especially those produced by *C. albicans*, have emerged as increasingly important causes of morbidity and mortality. Clinical and autopsy studies have confirmed the marked increase in the incidence of systemic candidiasis, at the same time reflecting an increase in the frequency of candidemia (2, 20). Candidiasis is the cause of more fatalities than any other systemic mycosis. Wey et al. (47) recently reported the enormous impact of systemic candidiasis on hospitalized patients. The crude mortality rate of patients with candidemia versus that of controls was reported to be 57 versus 19%, respectively, producing an attributable mortality rate of 38%. The same investigators also found that the mean length of stay of patients with candidemia was significantly greater (70 days versus 40 days for controls), resulting in an attributable excess length of stay of 30 days. The lack of a reliable and simple typing system for identification of *C. albicans* strain types has hampered the investigative efforts of most researchers in understanding the evolution of nosocomial *Candida* infection, avenues of dissemination, and strains more likely to cause invasive disease versus colonization.

Numerous typing systems for *C. albicans* have been used in the past; most have relied upon phenotypic variations in the species. These typing systems lack the sensitivity needed as epidemiologic markers and suffer from a lack of reproducibility, including problems of observer bias, and lack of confirmation by other methods. Some studies have lacked the epidemiologic information necessary for evaluation of the relatedness of isolates. Another common problem with the various typing systems has been strain clustering into only a few groups. With previous methods, most of the strains were congregated into two or three groups, resulting in a typing system that lacked the ability to differentiate different strains (45).

DNA analysis and REA have been used in the analysis of strain relatedness of diverse species of viruses and bacteria. In the last decade, molecular biology techniques have been used to analyze the genomic variations in yeast DNAs. Strains have been compared by using repetitive sequence analysis of ribosomal DNA, mitochondrial DNA polymorphisms, or cloned DNA probes (8, 13, 22, 42). Although these methods have greatly enhanced our knowledge of *C. albicans*, the problem of strain clustering into a few groups remains. In 1984, Schwartz and Cantor described the use of PFGE for analysis of large chromosomes greater than 2 Mb by using *Saccharomyces cerevisiae* as a model (37). Snell et al., by using the CHEF technique, were able to provide improved resolution of these large chromosomal bands (41). This was accomplished by eliminating the inhomogeneity of the electrical fields used for electrophoresis. Homogeneous electrical fields produced sharper and more distinct banding patterns. The patterns produced allowed electrophoretic karyotyping of the genomes of various yeasts, including *C. albicans*. REA was initially used by Scherer and Stevens (35, 36), who predicted that this method, because of its stability and reproducibility, would be useful for large-scale epidemiologic studies of *C. albicans*. To evaluate the sensitivity and specificity of REA further, in the present study we compared REA and CHEF techniques by using 35 clinical isolates from separate patients in a large tertiary care center. The isolates used in this study were from patients who were epidemiologically associated, as well as from patients in different hospitals. By using two restriction endonucleases, *Eco*RI and *Msp*I, we were able to distinguish 17 separate *C. albicans* strain types. Endonuclease *Msp*I was more useful than *Eco*RI. The restriction patterns observed with *Msp*I were sharper, more distinct, and easier to evaluate, thus providing greater ability for differentiation between clinical isolates. In most circumstances, *Eco*RI added no further sensitivity to that found with *Msp*I alone. This effect could possibly be due to the fact that *Eco*RI is a high-frequency cleavage restriction endonuclease, producing a large number of fragments more closely related in size, thereby making it more difficult to distinguish definite restriction patterns. *Msp*I, which appears to have fewer recognition sites in *C. albicans*, results in fewer bands and thus sharper and more distinct restriction patterns. As in prior studies, we also were able to distinguish three to four intense bands in our restriction patterns (22, 35, 36). These intensely staining bands are postulated to be repetitive sequences of *Candida* DNA. We found that strains could also be differentiated by using the largest chromosomal REA fragments. The variations in these patterns formed the basis of our strain groups. These variations in the restriction patterns are possibly due to heterozygosity of the ribosomal DNA genome. Unfortunately, as in prior studies using REA of whole-cell DNA, 66% of our isolates from patients were clustered into two groups (22, 35). Further differentiation of strain types was therefore evaluated. The need for greater differentiation of strain types of *C. albicans* prompted us to use PFGE, specifically, the CHEF technique, to analyze the electrophoretic karyotype variations in *C. albicans*. PFGE has been used previously in the electrophoretic karyotyping of various other yeasts, including *C. albicans*, *S. cerevisiae*, and *S. pombe*, and even in the analysis of human DNA and a number of unicellular organisms, such as plasmodia, trypanosomes, and leishmanias, with good results (15, 18, 19, 23, 26, 27, 44).

The results of this study demonstrate that the CHEF technique is more sensitive than REA, producing 23 different strain types from the 35 clinical isolates studied. The 23 strains had many similarities, generally conserving five of the chromosomal bands. Two chromosomal bands generally varied in size, making them the principle differentiators of strains. On occasion, a low-molecular-weight band also helped to distinguish between strain types. Not only is the sensitivity of CHEF an advantage in the differentiation of strains, but the procedure is reproducible and is much simpler and easier to perform than REA. Additionally, with CHEF the number of isolates prepared at one time can be doubled or tripled compare with REA. Reproducibility was demonstrated by repeated testing of the same isolates, which resulted in identical patterns. The results of this study also demonstrate that CHEF is a reliable method for differentiation of epidemiologically associated strains. This enables comparison of clinical isolates obtained several months apart without repeated lysis or insert preparation, thus saving time, effort, and resources. With CHEF, strain differentiation is much easier because fewer band numbers help eliminate the observer bias, which is an additional drawback of REA. The major disadvantages of CHEF compared with REA are the cost and the time required to do the procedure.

The results of this study demonstrate that CHEF is more sensitive and useful than REA as a typing method for differentiation of *C. albicans* strains. The present results are also in agreement with those of Magee et al., Merz et al., and Monod et al., who have previously used PFGE to investigate genomic DNAs from *Candida* spp. as a means of strain differentiation in epidemiologic studies (23, 26, 27). The CHEF technique appears to be superior to REA as a marker of strain identity for *C. albicans* clinical isolates. Further

epidemiologic studies are necessary to determine the most appropriate epidemiologic and clinical setting for its use; however, CHEF is a promising method for electophoretic karyotyping of yeast genomes. It provides a very convenient, rapid, and reproducible way to assign genes to chromosomes and monitor entire genomes for chromosomal variations.

ACKNOWLEDGMENT

This work was supported in part by the William Beaumont Hospital Research Institute.

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